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EXPRESSION OF UROKINASE PLASMINOGEN ACTIVATOR INHIBITORS

Abstract:

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A method for preparing a urokinase-type plasminogen activator inhibitor by expressing HuPA1-48 from yeast is disclosed. Data supplied from the esp@cenet database - Worldwide

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(54) Title: EXPRESSION OF UROKINASE PLASMINOGEN ACTIVATOR INHIBITORS (57) Abstract A method for preparing a urokinase-type plasminogen activator inhibitor by expressing HuPA ₁₋₄₈ from yeast is disclosed.		

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Expression of Urokinase Plasminogen Activator Inhibitors

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Description

Technical Field

This invention relates to the fields of cellular biology and protein expression. More particularly, the invention relates to peptide ligands of the urokinase plasminogen activator receptor, and methods for preparing the same.

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Background of the Invention

Urokinase-type plasminogen activator (uPA) is a multidomain serine protease, having a catalytic "B" chain (amino acids 144-411), and an amino-terminal fragment ("ATF", aa 1-143) consisting of a growth factor-like domain (4-43) and a kringle (aa 47-135). The uPA kringle appears to bind heparin, but not fibrin, lysine, or aminohexanoic acid. The growth factor-like domain bears some similarity to the structure of epidermal growth factor (EGF), and is thus also referred to as an "EGF-like" domain. The single chain pro-uPA is activated by plasmin, cleaving the chain into the two chain active form, which is linked together by a disulfide bond.

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uPA binds to its specific cell surface receptor (uPAR). The binding interaction is apparently mediated by the EGF-like domain (S.A. Rabbani *et al.*, J Biol Chem (1992) 267:14151-56). Cleavage of pro-uPA into active uPA is accelerated when pro-uPA and plasminogen are receptor-bound. Thus, plasmin activates pro-uPA, which in turn activates more plasmin by cleaving plasminogen. This positive feedback cycle is apparently limited to the receptor-based proteolysis on the cell surface, since a large excess of protease inhibitors is found in plasma, including α_2 antiplasmin, PAI-1 and PAI-2.

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Plasmin can activate or degrade extracellular proteins such as fibrinogen, fibronectin, and zymogens. Plasminogen activators thus can regulate extracellular proteolysis, fibrin clot lysis, tissue remodeling, developmental cell migration, inflammation, and metastasis. Accordingly, there is great interest in developing uPA inhibitors and uPA receptor antagonists. E. Appella *et al.*, J Biol Chem (1987) 262:4437-40 determined that receptor binding activity is localized in the EGF-like domain, and that residues 12-32 appear to be critical for binding. The critical domain alone (uPA₁₂₋₃₂) bound uPAR with an affinity of 40 nM (about 100 fold less than intact ATF).

10 S.A. Rabbani *et al.*, *supra*, disclosed that the EGF-like domain is fucosylated at Thr₁₈, and reported that fucosylated EGF-like domain (uPA₄₋₄₃, produced by cleavage from pro-uPA) was mitogenic for an osteosarcoma cell line, SaOS-2. In contrast, non-fucosylated EGF-like domain bound uPAR with an affinity equal to the fucosylated EGF-like domain, but exhibited no mitogenic activity. Non-fucosylated EGF-like domain competed for binding to uPAR with fucosylated EGF-like domain, and reduced the mitogenic activity observed. 15 Neither EGF-like domain was mitogenic in U937 fibroblast cells.

Previously, it was suggested that an "epitope library" might be made by cloning synthetic DNA that encodes random peptides into filamentous phage vectors (Parmley and Smith, Gene (1988) 73:305). It was proposed that the synthetic DNA be cloned into the coat protein gene III because of the likelihood of the encoded peptide becoming part of pIII without significantly interfering with pIII's function. It is known that the amino terminal half of pIII binds to the F pilus during infection of the phage into *E. coli*. It was suggested that such phage 20 that carry and express random peptides on their cell surface as part of pIII may provide a way of identifying the epitopes recognized by antibodies, particularly using antibody to affect the purification of phage from the library. Devlin, PCT WO91/18980 (incorporated herein by reference) described a method for producing a library consisting of random peptide sequences presented on filamentous 25

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phage. The library can be used for many purposes, including identifying and selecting peptides that have a particular bioactivity. An example of a ligand binding molecule would be a soluble or insoluble cellular receptor (*i.e.*, a membrane bound receptor), but would extend to virtually any molecule, including enzymes, that have the sought after binding activity. Description of a similar library is found in Dower *et al.*, WO91/19818. The present invention provides a method for screening such libraries (and other libraries of peptides) to determine bioactive peptides or compounds. Kang *et al.*, WO92/18619 disclosed a phage library prepared by inserting into the pVIII gene.

However, both the pIII and pVIII proteins are expressed in multiple copies in filamentous bacteriophage. As a result, the phage are selected and amplified based on their avidity for the target, rather than their affinity. To overcome this problem, a method for monovalent (only one test peptide per phage) phage display has been developed (H.B. Lowman *et al.*, Biochem (1991) 30:10832-38). To obtain monovalent display, the bacterial host is coinfectd with the phage library and a large excess of "helper" phage, which express only wild-type pIII (and/or pVIII) and are inefficiently packaged. By adjusting the ratio of display phage to helper phage, one can adjust the ratio of modified to wild-type display proteins so that most phage have only one modified protein. However, this results in a large amount of phage having only wild-type pIII (or pVIII), which significantly raises the background noise of the screening.

Disclosure of the Invention

One aspect of the invention is a method for producing non-fucosylated uPA EGF-like domain, particularly uPA₁₋₄₈.

Another aspect of the invention is non-fucosylated uPA₁₋₄₈, which is useful for inhibiting the mitogenic activity of uPA in cancer cells.

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Another aspect of the invention is a method for treating cancer and metastasis by administering an effective amount of a non-fucosylated uPA EGF-like domain, particularly uPA₁₋₄₈.

Another aspect of the invention is a method for pre-enriching a monovalent phage display mixture prior to screening for binding to a target, by providing a mixture of monovalent display phage and non-displaying phage, wherein the monovalent display phage display both a candidate peptide and a common peptide, the common peptide is identical for each monovalent display phage, and the candidate peptide is different for different monovalent display phage; and separating all phage displaying the common peptide from phage not displaying a common peptide.

Modes of Carrying Out The Invention

A. Definitions

The term "huPA" refers specifically to human urokinase-type plasminogen activator. The "EGF-like domain" is that portion of the huPA molecule responsible for mediating huPA binding to its receptor (uPAR). The EGF-like domain, sometimes called the growth factor-like domain ("GFD"), is located within the first 48 residues of huPA. The critical residues (essential for binding activity) have been localized to positions 12-32, although a peptide containing only those residues does not exhibit a binding affinity high enough to serve as a useful receptor antagonist.

The term "huPAR antagonist polypeptide" refers to a polypeptide having a sequence identical to the EGF-like domain of huPA (residues 1-48), or an active portion thereof. An "active portion" is one which lacks up to 10 amino acids, from the N-terminal or C-terminal ends, or a combination thereof, of the huPA₁₋₄₈ polypeptide, and exhibits a $K_d \leq 5$ nM with huPAR. The term "active analog" refers to a polypeptide differing from the sequence of the EGF-like domain of huPA₁₋₄₈ or an active portion thereof by 1-7 amino acids, but which

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still exhibits a $K_d \leq 5$ nM with huPAR. The differences are preferably conservative amino acid substitutions, in which an amino acid is replaced with another naturally-occurring amino acid of similar character. For example, the following substitutions are considered "conservative": Gly \leftrightarrow Ala; Val \leftrightarrow Ile \leftrightarrow Leu; Asp \leftrightarrow Glu; Lys \leftrightarrow Arg; Asn \leftrightarrow Gln; and Phe \leftrightarrow Trp \leftrightarrow Tyr. Nonconservative changes are generally substitutions of one of the above amino acids with an amino acid from a different group (e.g., substituting Asn for Glu), or substituting Cys, Met, His, or Pro for any of the above amino acids. The huPAR antagonist polypeptides of the invention should be substantially free of peptides derived from other portions of the huPA protein. For example, a huPAR antagonist composition should contain less than 20 wt% uPA B domain (dry weight, absent excipients), preferably less than 10 wt% uPA-B, more preferably less than 5 wt% uPA-B, most preferably no detectable amount. The huPAR antagonist polypeptides also preferably exclude the kringle region of uPA.

The term "expression vector" refers to an oligonucleotide which encodes the huPAR antagonist polypeptide of the invention and provides the sequences necessary for its expression in the selected host cell. Expression vectors will generally include a transcriptional promoter and terminator, or will provide for incorporation adjacent to an endogenous promoter. Expression vectors will usually be plasmids, further comprising an origin of replication and one or more selectable markers. However, expression vectors may alternatively be viral recombinants designed to infect the host, or integrating vectors designed to integrate at a preferred site within the host's genome. Expression vectors may further comprise an oligonucleotide encoding a signal leader polypeptide. When "operatively connected", the huPAR antagonist is expressed downstream and in frame with the signal leader, which then provides for secretion of the huPAR antagonist polypeptide by the host to the extracellular medium. Presently preferred signal leaders are the *Saccharomyces cerevisiae* α -factor leader (partic-

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ularly when modified to delete extraneous Glu-Ala sequences), and the ubiquitin leader (for intracellular expression).

The term "transcriptional promoter" refers to an oligonucleotide sequence which provides for regulation of the DNA → mRNA transcription process, typically based on temperature, or the presence or absence of metabolites, inhibitors, or inducers. Transcriptional promoters may be regulated (inducible/repressible) or constitutive. Yeast glycolytic enzyme promoters are capable of driving the transcription and expression of heterologous proteins to high levels, and are particularly preferred. The presently preferred promoter is the hybrid ADH2/GAP promoter described in Tekamp-Olson *et al.*, US 4,876,197 (incorporated herein by reference), comprising the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase promoter in combination with the *S. cerevisiae* alcohol dehydrogenase II upstream activation site.

The term "host" refers to a yeast cell suitable for expressing heterologous polypeptides. There are a variety of suitable genera, such as *Saccharomyces*, *Schizosaccharomyces*, *Kluveromyces*, *Pichia*, *Hansenula*, and the like. Presently preferred are yeast of the *Saccharomyces* genus, particularly *Saccharomyces cerevisiae*.

The term "uPA-mediated disorder" refers to a disease state or malady which is caused or exacerbated by a biological activity of uPA. The primary biological activity exhibited is plasminogen activation. Disorders mediated by plasminogen activation include, without limitation, inappropriate angiogenesis (*e.g.*, diabetic retinopathy, corneal angiogenesis, Kaposi's sarcoma, and the like), metastasis and invasion by tumor cells, and chronic inflammation (*e.g.*, rheumatoid arthritis, emphysema, and the like). Fucosylated ATF is also mitogenic for some tumor cells (*e.g.*, SaOS-2 osteosarcoma cells), which sometimes self-activate in an autocrine mechanism. Accordingly, the huPAR antagonist of the invention is effective in inhibiting the proliferation of uPA-activated tumor cells.

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The term "effective amount" refers to an amount of huPAR antagonist polypeptide sufficient to exhibit a detectable therapeutic effect. The therapeutic effect may include, for example, without limitation, inhibiting the growth of undesired tissue or malignant cells, inhibiting inappropriate angiogenesis, limiting tissue damage caused by chronic inflammation, and the like. The precise effective amount for a subject will depend upon the subject's size and health, the nature and severity of the condition to be treated, and the like. Thus, it is not possible to specify an exact effective amount in advance. However, the effective amount for a given situation can be determined by routine experimentation based on the information provided herein.

The term "pharmaceutically acceptable" refers to compounds and compositions which may be administered to mammals without undue toxicity. Exemplary pharmaceutically acceptable salts include mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like.

The term "pre-enriching" refers to increasing the concentration of candidate phage in a monovalent phage display mixture by removing phage which do not have a candidate peptide. A "monovalent phage display mixture" is a mixture of phage containing recombinant phage and helper phage in a ratio such that most phage display at most one recombinant surface protein.

The term "common peptide" refers to a distinctive heterologous (not wild-type) peptide sequence which is displayed identically by all recombinant members of a phage (or other host) library. The common peptide is preferably an epitope recognized by a high-affinity antibody, which is not cross-reactive with any epitopes naturally occurring in the wild-type phage. The common peptide permits one to select all recombinant phage (having a common peptide and a random candidate peptide) as a set, and purify them away from non-recombinant (wild-type) phage. The presently preferred common peptide is Glu-Tyr-Met-Pro-Met-Glu.

B. General Method

The present invention relies on the fact that yeast do not fucosylate proteins upon expression, but are able to express properly folded, active uPA and fragments. One may employ other eukaryotic hosts in the practice of the invention as long as the host is incapable of fucosylating proteins, whether naturally or due to manipulation (*e.g.*, genetic mutation or antibiotic treatment). Presently preferred hosts are yeasts, particularly *Saccharomyces*, *Schizosaccharomyces*, *Kluveromyces*, *Pichia*, *Hansenula*, and the like, especially *S. cerevisiae*. Strains AB110 and MB2-1 are presently preferred.

The expression vector is constructed according to known methods, and typically comprises a plasmid functional in the selected host. The uPA sequence used may be cloned following the method described in Example 1 below. Variations thereof (*i.e.*, active fragments and active analogs) may be generated by site-specific mutagenesis, imperfect PCR, and other methods known in the art. Stable plasmids generally require an origin of replication (such as the yeast 2μ ori), and one or more selectable markers (such as antibiotic resistance) which can be used to screen for transformants and force retention of the plasmid. The vector should provide a promoter which is functional in the selected host cell, preferably a promoter derived from yeast glycolytic enzyme promoters such as GAPDH, GAL, and ADH2. These promoters are highly efficient, and can be used to drive expression of heterologous proteins up to about 10% of the host cell weight. The presently preferred promoter is a hybrid ADH2/GAP promoter comprising the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase promoter in combination with the *S. cerevisiae* alcohol dehydrogenase II upstream activation site.

The expression vector should ideally provide a signal leader sequence between the promoter and the huPAR antagonist polypeptide sequence. The signal leader sequence provides for translocation of the huPAR antagonist polypeptide through the endoplasmic reticulum and export from the cell into the

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extracellular medium, where it may be easily harvested. There are a number of signal leader sequences known that are functional in yeast. The yeast α -factor leader is presently preferred (see U.S. 4,751,180, incorporated herein by reference).

5 Alternatively, the vector may provide for integration into the host genome, as is described by Shuster, PCT WO92/01800, incorporated herein by reference.

Transformations into yeast can be carried out according to the method of A. Hinnen *et al.*, Proc Natl Acad Sci USA (1978) 75:1929-33, or H. Ito *et al.*, J Bacteriol (1983) 153:163-68. After DNA is taken up by the host cell, the vector
10 integrates into the yeast genome at one or more sites homologous to its targeting sequence. It is presently preferred to linearize the vector by cleaving it within the targeting sequence using a restriction endonuclease, as this procedure increases the efficiency of integration.

15 Following successful transformations, the number of integrated sequences may be increased by classical genetic techniques. As the individual cell clones can carry integrated vectors at different locations, a genetic cross between two appropriate strains followed by sporulation and recovery of segregants can result in a new yeast strain having the integrated sequences of both
20 original parent strains. Continued cycles of this method with other integratively transformed strains can be used to further increase the copies of integrated plasmids in a yeast host strain. One may also amplify the integrated sequences by standard techniques, for example by treating the cells with increasing concentrations of copper ions (where a gene for copper resistance has been
25 included in the integrating vector).

Correct ligations for plasmid construction may be confirmed by first transforming *E. coli* strain MM294 obtained from *E. coli* Genetic Stock Center, CGSC #6135, or other suitable host with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or

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using other markers depending on the plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of D.B. Clewell *et al.*, Proc Natl Acad Sci USA (1969) 62:1159, optionally following chloramphenicol amplification (D.B. Clewell, J Bacteriol 5 (1972) 110:667). Isolated DNA is analyzed by restriction mapping and/or sequenced by the dideoxy method of F. Sanger *et al.*, Proc Natl Acad Sci USA (1977) 74:5463 as further described by Messing *et al.*, Nucl Acids Res (1981) 9:309, or by the method of Maxam and Gilbert, Meth Enzymol (1980) 65:499.

huPAR antagonist polypeptides may be assayed for activity by methods 10 known in the art. For example, one may assay competition of the antagonist against native uPA for cell surface receptor binding. Competition for the receptor correlates with inhibition of uPA biological activity. One may assay huPAR antagonist polypeptides for anti-mitogenic activity on appropriate tumor cell lines, such as the osteosarcoma cell line SaOS-2 described in the art. Inhibition of 15 mitogenic activity may be determined by comparing the uptake of ³H-T in osteosarcoma cells treated with the antagonist against controls. One may also assay huPAR antagonists for anti-invasive activity on appropriate tumor cell lines, such as HOC-1 and HCT116 (W. Schlechte *et al.*, Cancer Comm (1990) 2:173-79; H. Kobayashi *et al.*, Brit J Cancer (1993) 67:537-44).

20 huPAR antagonists are administered orally, topically, or by parenteral means, including subcutaneous and intramuscular injection, implantation of sustained release depots, intravenous injection, intranasal administration, and the like. When used to treat tumors, it may be advantageous to apply the huPAR antagonist directly to the site, *e.g.*, during surgery to remove the bulk of the 25 tumor. Accordingly, huPAR antagonist may be administered as a pharmaceutical composition comprising huPAR antagonist in combination with a pharmaceutically acceptable excipient. Such compositions may be aqueous solutions, emulsions, creams, ointments, suspensions, gels, liposomal suspensions, and the like. Suitable excipients include water, saline, Ringer's solution, dextrose solution, and

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solutions of ethanol, glucose, sucrose, dextran, mannose, mannitol, sorbitol, polyethylene glycol (PEG), phosphate, acetate, gelatin, collagen, Carbopol®, vegetable oils, and the like. One may additionally include suitable preservatives, stabilizers, antioxidants, antimicrobials, and buffering agents, for example, BHA, BHT, citric acid, ascorbic acid, tetracycline, and the like. Cream or ointment bases useful in formulation include lanolin, Silvadene® (Marion), Aquaphor® (Duke Laboratories), and the like. Other topical formulations include aerosols, bandages, and other wound dressings. Alternatively, one may incorporate or encapsulate the huPAR antagonist in a suitable polymer matrix or membrane, thus providing a sustained-release delivery device suitable for implantation near the site to be treated locally. Other devices include indwelling catheters and devices such as the Alzet® minipump. Ophthalmic preparations may be formulated using commercially available vehicles such as Sorbi-care® (Allergan), Neodecadron® (Merck, Sharp & Dohme), Lacrilube®, and the like, or may employ topical preparations such as that described in US 5,124,155, incorporated herein by reference. Further, one may provide a huPAR antagonist in solid form, especially as a lyophilized powder. Lyophilized formulations typically contain stabilizing and bulking agents, for example human serum albumin, sucrose, mannitol, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co.).

The amount of huPAR antagonist required to treat any particular disorder will of course vary depending upon the nature and severity of the disorder, the age and condition of the subject, and other factors readily determined by one of ordinary skill in the art. The appropriate dosage may be determined by one of ordinary skill by following the methods set forth below in the examples. As a general guide, about 0.01 mg/Kg to about 50 mg/Kg huPAR antagonist administered i.v. or subcutaneously is effective for inhibiting tissue damage due to chronic inflammation. For treating corneal angiogenesis, huPAR

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antagonist may be administered locally in a gel or matrix at a concentration of about 0.001 mg/Kg to about 5 mg/Kg.

C. Examples

5 The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

Example 1

10 (Cloning and Expression of huPA₁₋₄₈)

DNA encoding residues 1-48 of mature human uPA (huPA) was cloned into a yeast expression vector as a fusion with the yeast alpha-factor leader (α Fl), under transcriptional control of a hybrid ADH2-GAP promoter. The α Fl is described in Brake, US 4,870,008, incorporated herein by reference. The hybrid
15 ADH2-GAP promoter is described in Tekamp-Olson *et al.*, US 4,876,197, and Tekamp-Olson *et al.*, US 4,880,734, both incorporated herein by reference.

The gene encoding huPA was obtained by PCR using the following sense and nonsense primers:

20 5'-ATGCTAGATCTAATGAACTTCATCAGGTACCATCG-3' (SEQ ID NO:1), and

5'-CGATAGATCTTTATTTTGACTTATCTATTTACAG-3' (SEQ ID NO:2).

Each of the above primers introduces a BglII site at the ends for cloning into the expression vector. Additionally, the sense strand primer introduces a KpnI site
25 14 nucleotides downstream from the signal peptide cleavage site, and the nonsense strand primer introduces a stop codon after Lys at position 48. The template DNA used was a clone of full length mature huPA in a yeast expression vector, as an alpha-factor fusion (pAB24UK300, consisting of the yeast shuttle vector pAB24 having a cassette inserted at the BamHI site, the cassette containing

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the ADH2-GAP hybrid promoter, the yeast α -factor leader, the coding sequence for mature human uPA, and the GAP terminator, obtained from P. Valenzuela, Chiron Corporation) derived from a human kidney cDNA library (M.A. Truett *et al.*, DNA (1985) 4:333-49). Polymerase chain reactions were carried out in 100 μ L volumes with the following components: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 1 μ M each primer, 9 ng template plasmid, and 2.5 U *Taq* DNA polymerase. The reaction conditions were 94°C for 1 min, followed by 37°C for 2 min, then 72°C for 3 min, for 30 cycles. Both the PCR fragment and a subcloning vector (pCBR, described by Frederik *et al.*, J Biol Chem (1990) 265:3793) containing the yeast expression cassette were digested with BglII and then ligated together, after treatment of the pCBR vector with alkaline phosphatase. Once the subclone was obtained (pCBRuPA α 13), the expression cassette was isolated via BamHI digestion and ligated into the yeast shuttle vector (pAB24) to yield pAB24 α 13uPA1-48.

The expression plasmid was transformed into *Saccharomyces cerevisiae* AB110 (MAT α leu2-3 -112 ura3 -52 pep4 -3 [cir]^o) using the lithium acetate method (Ito *et al.*, J Bacteriol (1983) 153:163), and selected for uracil prototrophy. The plasmid copy number was then amplified by growth on minimal media without leucine, containing 8% glucose to keep ADH2-GAP promoter-mediated expression repressed. High level expression of secreted huPA₁₋₄₈ was obtained with pAB24 α 13uPA1-48 transformants of AB110 grown in leu⁻ medium and inoculating at 1:10 into YEP 4% glucose medium. All yeast cultures were grown at 30°C, 275 rpm, for 96 hours.

25

Example 2

(Purification of huPA₁₋₄₈)

One liter of yeast supernatant was harvested by centrifuging the cells at 2600 \times g. Protein was concentrated from the supernatant by adding 70%

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ammonium sulfate, incubating for 1 hr at 4°C, and separating the protein precipitate by centrifuging at 11,000 × g for 1 hr at 4°C. The protein pellets were resuspended in buffer containing 20 mM potassium phosphate, pH 7.0, 50 mM NaCl, and 1 mM EDTA. The suspension was dialyzed against the same
 5 buffer, with two changes of 4 L, overnight at 4°C. The entire dialysate was loaded onto a 1.8 L Sephadex® G-50 column at room temperature. Fractions were collected and monitored with UV at 254 nm, then pooled based on 16% Tris-Tricine SDS-PAGE (Novex) under non-reducing conditions. The peak
 10 fractions, containing monomeric huPA₁₋₄₈, were then loaded onto a 22 mm C18 reverse phase HPLC column (Vydac) and the protein eluted with a 0.6% gradient of acetonitrile containing 1% TFA. The major peak eluting at 33.5 minutes was collected and lyophilized. The purification yield is summarized in Table 1:

TABLE 1: Purification of huPA₁₋₄₈

15	Sample	Total Protein	Total Units ^b
	Yield		
	Crude supernatant	~ 200 mg ^a	3.3 × 10 ⁶ --
20	Ammonium sulfate	160 mg	2.0 × 10 ⁶ 60%
	G50 Column	103 mg	1.3 × 10 ⁶ 42%
	HPLC Purified	8.4 mg	7.4 × 10 ⁵ 22%

a) Estimated protein concentration due to interference with BCA assay

25 b) Unit = volume of crude sample required to inhibit binding of ¹²⁵I-ATF 50% in competition with biotinylated suPAR.

Example 3

30 (Characterization of huPA₁₋₄₈)

Purified huPA₁₋₄₈ was subjected to amino acid analysis and N-terminal sequencing, yielding the expected composition and sequence. The Edman

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degradation was performed through residue 20. A stoichiometric amount of threonine was observed at cycle 18, indicating that this residue was not modified by fucosylation, as is found for uPA purified from eukaryotic cells. The absence of post translational modification was later confirmed by electrospray mass spectrometry. The binding activity of the recombinant huPA₁₋₄₈ was determined using a radio-receptor binding assay.

Baculovirus-derived recombinant human urokinase receptor was expressed as a truncated, soluble molecule as described previously for mouse L-cells (Masucci *et al.*, J Biol Chem (1991) 266:8655). The purified receptor was biotinylated with NHS-biotin, and immobilized at 1 µg/mL in PBS/0.1 % BSA on streptavidin coated 96-well plates. Human uPA ATF (residues 1-135, obtained from M. Shuman, University of California, San Francisco) was iodinated using the Iodogen method (Pierce), and used as tracer. The ¹²⁵I-ATF was incubated at 100-500 pM with increasing amounts of huPA₁₋₄₈ in triplicate (100 pM - 1 µM) for 2 hours at room temperature in 0.1 % BSA/PBS in a total volume of 200 µL. The plates were then washed 3 times with PBS/BSA, and the remaining bound radioactivity determined. The apparent K_d observed for huPA₁₋₄₈ was 0.3 nM, comparable to that reported for ATF and intact uPA.

20

Example 4

(Construction of huPA₁₋₄₈ Muteins)

In order to efficiently analyze the features of huPA₁₋₄₈, we performed a series of mutagenesis experiments utilizing phage display. Attempts to employ the system described by Scott and Smith, Science (1990) 249:386-90, were not successful. However, the use of monovalent phage display, using a phagemid and helper phage as described by Lowman *et al.*, Biochem (1991) 30:10832-38, did result in functional display of the protein domain. Finally, we employed an affinity epitope "tag" to reduce the fraction of phage bearing only wild-type pIII protein, reducing the background in panning experiments.

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A.) Construction of Phagemids:

The starting materials were a phagemid construct (pGMEGF) comprising a human epidermal growth factor (hEGF) gene linked to the lac promoter, using pBluescript (Stratagene) as the backbone. The polylinker region of the vector
5 contained within a PvuII fragment was replaced by a cassette comprising a leader sequence from the photobacterial superoxide dismutase fused to a synthetic gene for hEGF, in turn fused to residues 198-406 of the M13 pIII gene. The sequence of the insert is shown in SEQ ID NO:3. A synthetic gene encoding human urokinase residues 1-48 was obtained from J. Stratton-Thomas, Chiron
10 Corporation.

Fusion proteins were generated using PCR. A first set of primers EUKMPCR1 and EUKGPCR1 were used with primer EUKPCR2 to add epitope tags to huPA₁₋₄₈ at the N-terminus, and to add an amber codon (TAG) and a BamHI site within residues 249-254 of the pIII protein at the C-terminus.
15 EUKMPCR1: CTCATCAAGCTTTAGCGGACTACAAAGACGATGACGATAAGAGCAATGAACTTCATCAAG (SEQ ID NO:5);
EUKGPCR1: CTCATCAAGCTTTAGCCGAATACATGCCAATGGAAAGCAATGAACTTCATCAAG (SEQ ID NO:6);
EUKPCR2: CACCGGAACCGGATCCACCCTATTTGACTTATC (SEQ ID
20 NO:7).
The PCR reactions yielded primary products of the expected sizes, 204 and 197 bp.

A second set of primers, SRO1 and EUKCPCR1, were used with the EGF-containing phagemid construct as template. These primers added a BamHI
25 site at pIII residues 250-251 and amplified a fragment ending at the unique ClaI site at residues 297-299 of pIII.
SRO1: GAAATAGATAAGTCAAAATAGGGTGGATCCGGTTCGGTGATTTGATTATG (SEQ ID NO:8); and
EUKCPCR1: GAAACCATCGATAGCAGCACCG (SEQ ID NO:9).

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This PCR reaction yielded a primary product of approximately 180 bp. The PCR reaction products were separated from unreacted primers by size exclusion chromatography (Chromaspin-100, Clontech), digested with restriction enzymes Hd3 and BamHI (set 1) or BamHI and Cla1 (set 2), and isolated from a 2.5 % agarose gel, using the Mermaid procedure (Bio-101). Each of the set 1 fragments were ligated with the C-terminal reaction 2 fragment, the ligations digested with Hd3 and Cla1, and the resulting fragments ligated into pGMEGF (digested with Hd3 and Cla1, dephosphorylated with alkaline phosphatase). The ligations were transformed into *E. coli* JS5 (Biorad) by electroporation. Strain JS5 overproduces lac repressor, and is sup0, preventing expression of the uPA₁₋₄₈-pIII fusion protein due to the amber stop codon between the uPA₁₋₄₈ and pIII genes. Correct clones were identified by restriction analysis and confirmed by DNA sequencing. These steps yielded phagemids pHM1a (M1Flag-uPA₁₋₄₈) and pHM3a (Glutag-uPA₁₋₄₈). The DNA sequences of the fusion proteins in these phagemids are shown in SEQ ID NO:10 and SEQ ID NO:12.

The phagemid containing a synthetic gene for uPA₁₋₄₈ was constructed in the same vector by the following steps. The sequence of the synthetic gene is shown in SEQ ID NO:14. Plasmid pCBRuPA (16 µg), a derivative of pCBR (Frederick *et al.*, *J Biol Chem* (1990) 265:3793) containing this synthetic gene for uPA₁₋₄₈, inserted between the yeast α-factor leader and GAPDH terminator as a BglII fragment, was digested with SacI and ClaI, and adapted for phagemid expression using the following set of synthetic oligonucleotides:

SRO35: AGCTTTAGCGGAATACATGCCAATGGAAAGCAATGAGCT (SEQ ID NO:16);

25 SRO36: CATTGCTTTCCATTGGCATGTATTCCGCTAA (SEQ ID NO:17);

SRO37: CGATAAGTCAAAATAGGGTG (SEQ ID NO:18); and

SRO38: GATCCACCCTATTTTGACTTAT (SEQ ID NO:19).

Oligonucleotides SRO36 and SRO37 (250 pmol) were phosphorylated with polynucleotide kinase and annealed with equimolar amounts of oligos SRO35 and

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SRO38, respectively. The two annealed duplexes (125 pmol) were ligated overnight with the digested plasmid DNA, the ligase heat inactivated, and the ends phosphorylated with polynucleotide kinase. The DNA was run on a 6% polyacrylamide gel and the correct sized band (ca. 200 bp) was excised and isolated. The insert was ligated with plasmid pHM1a (digested with Hd3 and BamHI) and phosphatased, and the ligations transformed into *E. coli* JS5. The correct recombinants were identified by restriction analysis, and confirmed by DNA sequencing, yielding phagemid pHM3-3.

B.) Production and Panning of Phagemids:

To produce phagemid particles, DNAs were transformed into *E. coli* strain XL1-blue (Stratagene) by electroporation. This strain was used because it is supE44 (TAG codon encodes Gln), lacI^Q (overproduces lac repressor), and makes phage (F' +). Overnight cultures were grown in 2× YT broth containing 50 µg/mL ampicillin and 10 µg/mL tetracycline (to maintain the F'). Cells were diluted 1:50 or 1:100 into the same media, grown for 20 minutes at 37°C for 10 minutes at 225 rpm to enhance phage attachment, and then grown with normal agitation at 325 rpm overnight. Phage particles were then purified and concentrated by two successive precipitations with polyethylene glycol. The concentrations of phage present were determined by infection of *E. coli* XL1-blue and plating on L broth plates containing 50 µg/mL ampicillin.

To pan for binding phage particles, small tissue culture plates were coated either with anti-Glu antibody (R. Clark, Onyx Corporation) or streptavidin at 10 µg/mL in PBS overnight. Plates were then blocked with PBS containing 0.1% BSA. To the streptavidin plates was then added 1 µg/mL of biotinylated secreted human urokinase receptor obtained by recombinant baculovirus infection of *A. californica* Sf9 cells. After 2 hours at room temperature, the plates were again blocked with BSA, and phage (10⁶-10¹⁰ cfu) were added in 1 mL of PBS/BSA. After incubation for 1 hour, non-specifically adhered phage were removed by washing (7× 1 mL PBS/BSA), and the remaining phage eluted with

- 19 -

1 mL of 0.1 M glycine, pH 2.2, for 30 minutes. The eluted phage were immediately neutralized with 1 M Tris, pH 9.4, and stored at 4°C overnight. The number of phage eluted was determined by titring on *E. coli* XL1-blue on ampicillin plates. The procedure, where phage are first bound and eluted from the Glu-Ab plates and then panned against receptor plates, reduces the high background that would otherwise result from the large number of phage containing only wild type pIII: only phage containing an insert in pIII display an epitope tag and are selected on anti-Glu MAbs plates.

Table 2 shows that phagemids displaying uPA₁₋₄₈ are specifically bound and eluted from immobilized urokinase receptor. Table 3 demonstrates that the phagemid which displays a Glu tag-uPA₁₋₄₈ fusion is specifically retained by immobilized Glu Ab. Finally, Table 4 shows that a population of the Glu-uPA₁₋₄₈ phagemid which has been specifically eluted from the Glu Ab plates, is retained with a much higher yield on urokinase receptor plates, than is the unenriched phagemid population.

TABLE 2: Panning on Immobilized Receptor

Sample	Phage/phagemid	Input ^e	%Yield	
			-uPAR	+uPAR
1 ^a	1a	9.4×10^9	0.0018	0.094
2 ^b	3a	1.4×10^{10}	0.0014	0.08
3 ^c	pGMEGF	1.3×10^{10}	0.0015	0.0012
4 ^d	LP67 (control)	1.4×10^9	-	0.0099

^a M1-FLAG-UPAELD-short pIII (pHM1a)

^b Glu-tag-UPAELD-short pIII (pHM3a)

^c M1-FLAG-EGF-medium long pIII (pGMEGF)

^d LP67 - control phage (Amp^r M13)

^e ampicillin resistant colonies, in cfu

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TABLE 3: Panning phage with Glu-Ab or suPAR

Sample	Phage/phagemid	Input ^a	% Yield	
			suPAR ^b	GluAb
1	pHM1a	1.5×10^{10}	0.55 %	0.003 %
2	pHM3a	2.5×10^{10}	0.44 %	0.048 %
3	LP67 (control)	3.5×10^5	0.008 %	-

^a ampicillin resistant colonies, in cfu^b soluble uPA receptor

TABLE 4: Panning GluAb-unenriched and enriched phage on suPAR

Sample	Phage/phagemid	Input ^a	% Yield	
			suPAR ^b	GluAb
1	pHM3a	2.7×10^7	0.85 %	0.08 %
2	pHM3a (enriched)	6×10^6	9.7 %	3.3 %
3	LP67 (control)	5.4×10^6	<0.04 %	<0.02 %

^a ampicillin resistant colonies, in cfu^b soluble uPA receptor

These enriched phagemid pools are used for multiple mutagenesis strategies in order to identify improved uPA₁₋₄₈ ligands with altered specificity or improved affinity. For example the region between residues 13 and 32 of human uPA has been implicated in receptor binding (E. Appella *et al.*, *J Biol Chem* (1987) 262:4437-40). Key residues in the region from 19-30 can be easily mutated by replacing the region between the unique restriction sites KpnI and MunI.

In order to rapidly and quantitatively assess the binding affinities of the resulting uPA₁₋₄₈ variants, relatively large quantities of properly folded proteins are required. Although this could be done by bacterial expression, using the phagemid constructs in a sup0 strain and inducing with IPTG, such a strategy yields relatively small amounts of protein in the periplasm. A second strategy is to express the variants in yeast, as described above for the wild type protein. To accomplish this we have constructed a yeast expression vector which enables us to move fragments encoding residues 4-48 of uPA₁₋₄₈ in a single step from the phagemid vectors. This was accomplished as follows: Plasmid pAGαG,

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identical to pCBR except for a small deletion of an Xba fragment in the ADH2-GAPDH promoter, was digested with SacI, which cleaves once within the promoter, and then treated with Mung Bean nuclease which destroys the site. Subsequent religation yielded plasmid pAG α G-Sac. Digestion with BglII and treatment with alkaline phosphatase yielded a vector into which was ligated the BglII fragment corresponding to the synthetic gene for uPA₁₋₄₈. Transformation of *E. coli* strain HB101 to ampicillin resistance and restriction analysis yielded the correct clone. The 2.4 kB BamHI fragment from this plasmid (pAG α G-SacI-48synth), containing the expression cassette, was isolated and ligated into pAB24, which had been treated with BamHI and alkaline phosphatase. The resulting plasmid has unique SacI and XhoI sites which can be used for transfer of the phagemid 1-48 genes. This is accomplished by digesting the phagemid with BamHI, treating with Mung Bean Nuclease, digesting with SacI and isolating the 145 bp fragment. The vector is digested with XhoI, treated with Mung Bean Nuclease, digested with SacI, and treated with alkaline phosphatase. Ligation then yields the correct recombinants in a single step in the yeast expression vector. Transformation of yeast strain AB110 then yields high levels of secreted 1-48 variants for analysis.

Using this construct, one can express a library of uPA variations for screening. Variations may be constructed by a variety of methods, including low-fidelity PCR (which introduces a large number of random point mutations), site-specific mutation, primer-based mutagenesis, and ligation of the uPA₁₋₄₈ sequence (or portions thereof) to a random oligonucleotide sequence (*e.g.*, by attaching (NNS)_x to the uPA₁₋₄₈ coding sequence, or substituting NNS for one or more uPA₁₋₄₈ codons). Generation of random oligonucleotide sequences is detailed in Devlin, WO91/18980, incorporated herein by reference. Phage displaying uPA₁₋₄₈ variants (having one or more amino acid substitutions) are screened according to the protocol described above (using, *e.g.*, pHM3a as a positive control) and selected for improved binding.

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Example 5(Formulation of huPA₁₋₄₈)

huPA₁₋₄₈ formulations suitable for use in chemotherapy are prepared as follows:

5 A) Injectable Formulation:

	huPA ₁₋₄₈	7.0 mg
	Na ₂ HPO ₄ (0.5 M)	0.5 mL
	mannitol (25 %)	2.5 mL
	sodium laureate (1 %)	2.5 mL
10	pH	7.5
	<u>PBS qs</u>	<u>20 mL</u>

This formulation is prepared following the procedure set forth in US 4,816,440, incorporated herein by reference. The formulation is administered by
15 parenteral injection at the site to be treated. The formulation is also generally suitable for administration as eyedrops directly to the conjunctiva, or by intranasal administration as an aerosol. Alternatively, a concentrated formulation (*e.g.*, reducing the phosphate buffered saline to 2 mL) may be used to fill an Alzet® minipump, and the minipump implanted at the site to be treated.

20 B) Ophthalmic Preparation:

	huPA ₁₋₄₈	1 mg
	fibronectin	69 mg
	albumin	37.5 mg
	water	qs
25	<u>HCl (0.01 M)</u>	<u>qs</u>
		pH 4.0

This dosage form is prepared following the procedure set forth in US 5,124,155, incorporated herein by reference. The fibronectin and albumin are dissolved in water to form a 3.0 mL solution, and HCl added to a pH of 4.0,
30 causing the fibronectin to flocculate. The flocculent is filtered, and combined with the huPA₁₋₄₈. The mixture is then placed in a contact lens mold, and the mold closed for 30 min to form a corneal "shield" in the shape of a contact lens.

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The shield releases huPA₁₋₄₈ over a period of time, and is useful for preventing angiogenesis of corneal tissue following ophthalmic surgery.

5 The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in the art without departing from the spirit and the scope of the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Rosenberg, Steven
Stratton-Thomas, Jennifer R.
- 10 (ii) TITLE OF INVENTION: Expression of Urokinase Plasminogen
Activator Inhibitors
- (iii) NUMBER OF SEQUENCES: 19
- 15 (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Chiron Corporation
(B) STREET: 4560 Horton Street
(C) CITY: Emeryville
(D) STATE: CA
(E) COUNTRY: USA
20 (F) ZIP: 94608
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
25 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
30 (B) FILING DATE:
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
35 (A) NAME: Green, Grant D.
(B) REGISTRATION NUMBER: 31,259
(C) REFERENCE/DOCKET NUMBER: 0939.001
- (ix) TELECOMMUNICATION INFORMATION:
40 (A) TELEPHONE: 510-601-2706
(B) TELEFAX: 510-655-3542

(2) INFORMATION FOR SEQ ID NO:1:

- 45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
50 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- 55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- 60 ATGCTAGATC TAATGAACTT CATCAGGTAC CATCG
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- 25 -

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 35 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGATAGATCT TTATTTTGAC TTATCTATTT CACAG
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(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 953 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:

(B) CLONE: M1Flag-EGF-pIII fusion

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 25..903

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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- 26 -

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- 27 -

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 20 (2) INFORMATION FOR SEQ ID NO:4:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 293 amino acids
 (B) TYPE: amino acid
 25 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: protein
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
 30 Met Asn Lys Ala Lys Thr Leu Leu Phe Thr Ala Leu Ala Phe Gly Leu
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 35 Ser His Gln Ala Leu Ala Asp Tyr Lys Asp Asp Asp Asp Lys Asn Ser
 20 25 30
 Asp Ser Glu Cys Pro Leu Ser His Asp Gly Tyr Cys Leu His Asp Gly
 35 40 45
 40 Val Cys Met Tyr Ile Glu Ala Leu Asp Lys Tyr Ala Cys Asn Cys Val
 50 55 60
 45 Val Gly Tyr Ile Gly Glu Arg Cys Gln Tyr Arg Asp Leu Lys Trp Trp
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 50 Leu Pro Gln Pro Pro Val Asn Ala Gly Gly Gly Ser Gly Gly Gly Ser
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 115 120 125
 55 Gly Gly Ser Glu Gly Gly Gly Ser Gly Gly Gly Ser Gly Ser Gly Asp
 130 135 140
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 60 Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp

- 28 -

[illegible]

(2) INFORMATION FOR SEQ ID NO:5:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

40 (vii) IMMEDIATE SOURCE:
(B) CLONE: EUKMPCR1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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50 (2) INFORMATION FOR SEQ ID NO:6:

55 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

60 (iii) HYPOTHETICAL: NO

- 29 -

(vii) IMMEDIATE SOURCE:
(B) CLONE: EUKGPCR1

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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10 (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
15 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20 (iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:
(B) CLONE: EUKPCR2

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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30 34

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
35 (A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

45 (vii) IMMEDIATE SOURCE:
(B) CLONE: SRO1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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(2) INFORMATION FOR SEQ ID NO:9:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
60 (D) TOPOLOGY: linear

- 30 -

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

5 (vii) IMMEDIATE SOURCE:
(B) CLONE: EUKCPCR1

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
GAAACCATCG ATAGCAGCAC CG
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15 (2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 779 base pairs
(B) TYPE: nucleic acid
20 (C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(vii) IMMEDIATE SOURCE:
(B) CLONE: M1Flag uPA1-48 - pIII fusion
30 (ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 25..729
35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
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40 Met Asn Lys Ala Lys Thr Leu Leu Phe
1 5
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10 15 20 25
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50 Asp Asp Asp Asp Lys Ser Asn Glu Leu His Gln Val Pro Ser Asn Cys
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45 50 55
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243
60 Ile His Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys Glu
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- 31 -

5 ATA GAT AAG TCA AAA TAG GGT GGA TCC GGT TCC GGT GAT TTT GAT TAT
 291
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 90 95 100 105
 15 GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT
 387
 Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala
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 20 ACT GAT TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC
 435
 Thr Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly
 125 130 135
 25 CTT GCT AAT GGT AAT GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC
 483
 Leu Ala Asn Gly Asn Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser
 140 145 150
 30 CAA ATG GCT CAA GTC GGT GAC GGT GAT AAT TCA CCT TTA ATG AAT AAT
 531
 Gln Met Ala Gln Val Gly Asp Gly Asp Asn Ser Pro Leu Met Asn Asn
 155 160 165
 35 TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA TGT CGC CCT
 579
 Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu Cys Arg Pro
 170 175 180 185
 40 TTT GTC TTT AGC GCT GGT AAA CCA TAT GAA TTT TCT ATT GAT TGT GAC
 627
 Phe Val Phe Ser Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp
 190 195 200
 45 AAA ATA AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC
 675
 Lys Ile Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala
 205 210 215
 50 ACC TTT ATG TAT GTA TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG
 723
 Thr Phe Met Tyr Val Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys
 220 225 230
 55 GAG TCT TAATCATGCG CGCTCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA
 779
 Glu Ser
 235

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

60 (A) LENGTH: 235 amino acids
 (B) TYPE: amino acid

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

Met Asn Lys Ala Lys Thr Leu Leu Phe Thr Ala Leu Ala Phe Gly Leu
 1           5           10           15
10 Ser His Gln Ala Leu Ala Asp Tyr Lys Asp Asp Asp Asp Lys Ser Asn
    20           25           30
    Glu Leu His Gln Val Pro Ser Asn Cys Asp Cys Leu Asn Gly Gly Thr
    35           40           45
15 Cys Val Ser Asn Lys Tyr Phe Ser Asn Ile His Trp Cys Asn Cys Pro
    50           55           60
20 Lys Lys Phe Gly Gly Gln His Cys Glu Ile Asp Lys Ser Lys * Gly
    65           70           75           80
    Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn
    85           90           95
25 Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp
    100          105          110
    Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile
    115          120          125
30 Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly Ala
    130          135          140
    Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp
    145          150          155          160
    Gly Asp Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser
    165          170          175
40 Leu Pro Gln Ser Val Glu Cys Arg Pro Phe Val Phe Ser Ala Gly Lys
    180          185          190
    Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly
    195          200          205
45 Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe Ser
    210          215          220
    Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser
    225          230          235

```

(2) INFORMATION FOR SEQ ID NO:12:

```

55 (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 773 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: double
    (D) TOPOLOGY: linear

```

60 (ii) MOLECULE TYPE: cDNA

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(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:

5 (B) CLONE: Glu-tag uPA1-48 - pIII fusion

(ix) FEATURE:

(A) NAME/KEY: CDS

10 (B) LOCATION: 25..723

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

15 CCATGGCTAC AGAGGAATAT TAAA ATG AAT AAG GCA AAA ACT TTA CTC TTC
 51 Met Asn Lys Ala Lys Thr Leu Leu Phe
 1 Thr 5
 20 ACT GCG CTA GCT TTT GGT TTA TCT CAT CAA GCT TTA GCC GAA TAC ATG
 99 Thr Ala Leu Ala Phe Gly Leu Ser His Gln Ala Leu Ala Glu Tyr Met
 10 15 20 25
 25 CCA ATG GAA AGC AAT GAA CTT CAT CAA GTT CCA TCG AAC TGT GAC TGT
 147 Pro Met Glu Ser Asn Glu Leu His Gln Val Pro Ser Asn Cys Asp Cys
 30 35 40
 30 CTA AAT GGA GGA ACA TGT GTG TCC AAC AAG TAC TTC TCC AAC ATT CAC
 195 Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser Asn Ile His
 45 50 55
 35 TGG TGC AAC TGC CCA AAG AAA TTC GGA GGG CAG CAC TGT GAA ATA GAT
 243 Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys Glu Ile Asp
 60 65 70
 40 AAG TCA AAA TAG GGT GGA TCC GGT TCC GGT GAT TTT GAT TAT GAA AAG
 291 Lys Ser Lys * Gly Gly Ser Gly Ser Gly Asp Phe Asp Tyr Glu Lys
 75 80 85
 45 ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA AAT GCC GAT GAA AAC
 339 Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn Ala Asp Glu Asn
 90 95 100 105
 50 GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT GAT
 387 Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp
 110 115 120
 55 TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT
 435 Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala
 125 130 135
 60 AAT GGT AAT GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG
 483 Asn Gly Asn Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met

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	140	145	150
	GCT CAA GTC GGT GAC GGT GAT AAT TCA CCT TTA ATG AAT AAT TTC CGT		
5	531 Ala Gln Val Gly Asp Gly Asp Asn Ser Pro Leu Met Asn Asn Phe Arg	160	165
	CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA TGT CGC CCT TTT GTC		
10	579 Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu Cys Arg Pro Phe Val	175	180 185
	TTT AGC GCT GGT AAA CCA TAT GAA TTT TCT ATT GAT TGT GAC AAA ATA		
15	627 Phe Ser Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile	190	195 200
	AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT		
20	675 Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe	205	210 215
	ATG TAT GTA TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT		
25	723 Met Tyr Val Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser	220 225	230
	TAATCATGCG CGCTCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA		
30	773		

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 233 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met	Asn	Lys	Ala	Lys	Thr	Leu	Leu	Phe	Thr	Ala	Leu	Ala	Phe	Gly	Leu
1				5					10					15	
Ser	His	Gln	Ala	Leu	Ala	Glu	Tyr	Met	Pro	Met	Glu	Ser	Asn	Glu	Leu
			20					25					30		
His	Gln	Val	Pro	Ser	Asn	Cys	Asp	Cys	Leu	Asn	Gly	Gly	Thr	Cys	Val
		35					40					45			
Ser	Asn	Lys	Tyr	Phe	Ser	Asn	Ile	His	Trp	Cys	Asn	Cys	Pro	Lys	Lys
	50					55					60				
Phe	Gly	Gly	Gln	His	Cys	Glu	Ile	Asp	Lys	Ser	Lys	*	Gly	Gly	Ser
	65				70				75						80
Gly	Ser	Gly	Asp	Phe	Asp	Tyr	Glu	Lys	Met	Ala	Asn	Ala	Asn	Lys	Gly
			85						90					95	
Ala	Met	Thr	Glu	Asn	Ala	Asp	Glu	Asn	Ala	Leu	Gln	Ser	Asp	Ala	Lys

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	100	105	110
	Gly Lys Leu Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile Asp Gly		
	115	120	125
5	Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly Ala Thr Gly		
	130	135	140
10	Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp		
	145	150	155
	Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro		
	165	170	175
15	Gln Ser Val Glu Cys Arg Pro Phe Val Phe Ser Ala Gly Lys Pro Tyr		
	180	185	190
	Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly Val Phe		
	195	200	205
20	Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe Ser Thr Phe		
	210	215	220
25	Ala Asn Ile Leu Arg Asn Lys Glu Ser		
	225	230	

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
- 30 (A) LENGTH: 773 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
- 35 (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- 40 (vii) IMMEDIATE SOURCE:
(B) CLONE: Glu-tag uPA1-48 synth. - pIII map
- (ix) FEATURE:
- 45 (A) NAME/KEY: CDS
(B) LOCATION: 25..723

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

50	CCATGGCTAC AGAGGAATAT TAAA ATG AAT AAG GCA AAA ACT TTA CTC TTC
	51
	Met Asn Lys Ala Lys Thr Leu Leu Phe
	1 5
55	ACT GCG CTA GCT TTT GGT TTA TCT CAT CAA GCT TTA GCG GAA TAC ATG
	99
	Thr Ala Leu Ala Phe Gly Leu Ser His Gln Ala Leu Ala Glu Tyr Met
	10 15 20 25
60	CCA ATG GAA AGC AAT GAG CTC CAT CAA GTA CCA TCG AAC TGT GAC TGT
	147

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	Pro	Met	Glu	Ser	Asn	Glu	Leu	His	Gln	Val	Pro	Ser	Asn	Cys	Asp	Cys
					30					35					40	
5	CTA	AAT	GGA	GGT	ACC	TGT	GTG	TCC	AAC	AAG	TAC	TTT	TCG	AAC	ATT	CAC
	195															
	Leu	Asn	Gly	Gly	Thr	Cys	Val	Ser	Asn	Lys	Tyr	Phe	Ser	Asn	Ile	His
				45					50					55		
10	TGG	TGC	AAT	TGC	CCA	AAG	AAA	TTC	GGA	GGG	CAG	CAC	TGT	GAA	ATC	GAT
	243															
	Trp	Cys	Asn	Cys	Pro	Lys	Lys	Phe	Gly	Gly	Gln	His	Cys	Glu	Ile	Asp
			60					65					70			
15	AAG	TCA	AAA	TAG	GGT	GGA	TCC	GGT	TCC	GGT	GAT	TTT	GAT	TAT	GAA	AAG
	291															
	Lys	Ser	Lys	*	Gly	Gly	Ser	Gly	Ser	Gly	Asp	Phe	Asp	Tyr	Glu	Lys
		75					80					85				
20	ATG	GCA	AAC	GCT	AAT	AAG	GGG	GCT	ATG	ACC	GAA	AAT	GCC	GAT	GAA	AAC
	339															
	Met	Ala	Asn	Ala	Asn	Lys	Gly	Ala	Met	Thr	Glu	Asn	Ala	Asp	Glu	Asn
	90					95					100				105	
25	GCG	CTA	CAG	TCT	GAC	GCT	AAA	GGC	AAA	CTT	GAT	TCT	GTC	GCT	ACT	GAT
	387															
	Ala	Leu	Gln	Ser	Asp	Ala	Lys	Gly	Lys	Leu	Asp	Ser	Val	Ala	Thr	Asp
					110					115					120	
30	TAC	GGT	GCT	GCT	ATC	GAT	GGT	TTC	ATT	GGT	GAC	GTT	TCC	GGC	CTT	GCT
	435															
	Tyr	Gly	Ala	Ala	Ile	Asp	Gly	Phe	Ile	Gly	Asp	Val	Ser	Gly	Leu	Ala
				125					130					135		
35	AAT	GGT	AAT	GGT	GCT	ACT	GGT	GAT	TTT	GCT	GGC	TCT	AAT	TCC	CAA	ATG
	483															
	Asn	Gly	Asn	Gly	Ala	Thr	Gly	Asp	Phe	Ala	Gly	Ser	Asn	Ser	Gln	Met
			140					145					150			
40	GCT	CAA	GTC	GGT	GAC	GGT	GAT	AAT	TCA	CCT	TTA	ATG	AAT	AAT	TTC	CGT
	531															
	Ala	Gln	Val	Gly	Asp	Gly	Asp	Asn	Ser	Pro	Leu	Met	Asn	Asn	Phe	Arg
		155					160					165				
45	CAA	TAT	TTA	CCT	TCC	CTC	CCT	CAA	TCG	GTT	GAA	TGT	CGC	CCT	TTT	GTC
	579															
	Gln	Tyr	Leu	Pro	Ser	Leu	Pro	Gln	Ser	Val	Glu	Cys	Arg	Pro	Phe	Val
	170					175					180				185	
50	TTT	AGC	GCT	GGT	AAA	CCA	TAT	GAA	TTT	TCT	ATT	GAT	TGT	GAC	AAA	ATA
	627															
	Phe	Ser	Ala	Gly	Lys	Pro	Tyr	Glu	Phe	Ser	Ile	Asp	Cys	Asp	Lys	Ile
					190					195					200	
55	AAC	TTA	TTC	CGT	GGT	GTC	TTT	GCG	TTT	CTT	TTA	TAT	GTT	GCC	ACC	TTT
	675															
	Asn	Leu	Phe	Arg	Gly	Val	Phe	Ala	Phe	Leu	Leu	Tyr	Val	Ala	Thr	Phe
				205					210					215		
60	ATG	TAT	GTA	TTT	TCT	ACG	TTT	GCT	AAC	ATA	CTG	CGT	AAT	AAG	GAG	TCT
	723															
	Met	Tyr	Val	Phe	Ser	Thr	Phe	Ala	Asn	Ile	Leu	Arg	Asn	Lys	Glu	Ser

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220

225

230

TAATCATGCG CGCTCACTGG CCGTCGTTTT ACAACGTCGT GACTGGGAAA
773

5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 233 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: protein

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Asn Lys Ala Lys Thr Leu Leu Phe Thr Ala Leu Ala Phe Gly Leu
1 5 10 15
Ser His Gln Ala Leu Ala Glu Tyr Met Pro Met Glu Ser Asn Glu Leu
20 25 30
His Gln Val Pro Ser Asn Cys Asp Cys Leu Asn Gly Gly Thr Cys Val
25 35 40 45
Ser Asn Lys Tyr Phe Ser Asn Ile His Trp Cys Asn Cys Pro Lys Lys
50 55 60
Phe Gly Gly Gln His Cys Glu Ile Asp Lys Ser Lys * Gly Gly Ser
30 65 70 75 80
Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly
85 90 95
Ala Met Thr Glu Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys
35 100 105 110
Gly Lys Leu Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile Asp Gly
40 115 120 125
Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly Ala Thr Gly
130 135 140
Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp
45 145 150 155 160
Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro
50 165 170 175
Gln Ser Val Glu Cys Arg Pro Phe Val Phe Ser Ala Gly Lys Pro Tyr
180 185 190
Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg Gly Val Phe
55 195 200 205
Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe Ser Thr Phe
210 215 220
Ala Asn Ile Leu Arg Asn Lys Glu Ser
60 225 230

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(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:

15

(B) CLONE: SRO35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

20 AGCTTTAGCG GAATACATGC CAATGGAAAG CAATGAGCT
39

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

35

(vii) IMMEDIATE SOURCE:

(B) CLONE: SRO36

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

40 CATTGCTTTC CATTGGCATG TATTCCGCTA A
31

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- 45 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: cDNA

55

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:

60

(B) CLONE: SRO37

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGATAAGTCA AAATAGGGTG
20

5

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(iii) HYPOTHETICAL: NO

(vii) IMMEDIATE SOURCE:

20

(B) CLONE: SRO38

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

25 GATCCACCCT ATTTGACTT AT
22

- 40 -

WHAT IS CLAIMED:

1. A method for producing a non-fucosylated polypeptide consisting essentially of the EGF-like domain of human urokinase-type plasminogen activator, said method comprising:
- 5 providing a yeast host transformed with an expression vector, said vector comprising a transcriptional promoter operably linked to an oligonucleotide encoding a huPAR antagonist polypeptide consisting essentially of the EGF-like domain of human urokinase-type plasminogen activator or an active analog
- 10 thereof;
- culturing said yeast host under conditions which promote expression of said polypeptide; and
- isolating said polypeptide.
- 15 2. The method of claim 1, wherein said oligonucleotide further encodes a signal leader polypeptide operatively connected to said huPAR antagonist polypeptide or analog, operative in said host cell to direct secretion of the expressed polypeptide.
- 20 3. The method of claim 2, wherein said signal leader comprises yeast α -factor leader.
4. The method of claim 3 wherein said yeast α -factor leader is *S. cerevisiae* α -factor leader.
- 25 5. The method of claim 1, wherein said host cell is *Saccharomyces cerevisiae*.

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6. The method of claim 1, wherein said huPAR antagonist polypeptide consists essentially of huPA₁₋₄₈.

7. A huPAR antagonist polypeptide composition comprising a non-fucosylated polypeptide consisting essentially of the EGF-like domain of human urokinase-type plasminogen activator or an active analog thereof.

8. The composition of claim 7, wherein said non-fucosylated polypeptide consists essentially of huPA₁₋₄₈.

10

9. The composition of claim 7, further comprising a pharmaceutically acceptable excipient.

10. A method for treating a uPA-mediated disorder, said method comprising:

15

providing a composition comprising a non-fucosylated polypeptide consisting essentially of the EGF-like domain of human urokinase-type plasminogen activator or an active analog thereof; and

administering an effective amount of said composition to a patient having a uPA-mediated disorder.

20

11. The method of claim 10, wherein said polypeptide consists essentially of huPA₁₋₄₈.

12. The method of claim 10, wherein said uPA-mediated disorder is selected from the group consisting of metastasis, inappropriate angiogenesis, and chronic inflammation.

25

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13. The method of claim 12, wherein said uPA-mediated disorder is selected from the group consisting of Kaposi's sarcoma, diabetic retinopathy, and rheumatoid arthritis.

5 14. The method of claim 10, wherein said composition is administered by instillation in the eye.

15. A method for pre-enriching a monovalent phage display mixture prior to screening for binding to a target, comprising:

- 10 (a) providing a mixture of monovalent display phage and non-displaying phage, wherein said monovalent display phage display both a candidate peptide and a common peptide, wherein said common peptide is identical for each monovalent display phage, and wherein said candidate peptide is different for different monovalent display phage; and
- 15 (b) separating all phage displaying a common peptide from phage not displaying a common peptide.

16. The method of claim 15, wherein said candidate peptide is huPA₁₋₄₈ or an active analog or active portion thereof.

20

17. The method of claim 15, wherein said common peptide comprises an antibody epitope.

18. The method of claim 17, wherein said epitope comprises Glu-Tyr-Met-Pro-Met-Glu.

25

19. The method of claim 15, further comprising:

- (c) contacting said separated phage displaying said common peptide with said target; and

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(d) separating phage which bind said target from phage which do not bind said target.

20. The method of claim 19, wherein said target comprises
5 huPAR.